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Characterization of OXA-204, a Carbapenem-Hydrolyzing Class D β-Lactamase from *Klebsiella pneumoniae*

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A *Klebsiella pneumoniae* clinical isolate recovered in Tunisia showed resistance to all β-lactams and decreased susceptibility to carbapenems. *K. pneumoniae* 204 expressed the carbapenem-hydrolyzing β-lactamase OXA-204, differing from OXA-48 by two amino acid substitutions (Gln98His and Thr99Arg) (class D β-lactamase [DBL] numbering). OXA-48 and OXA-204 shared similar resistance profiles, hydrolyzing carbapenems but sparing broad-spectrum cephalosporins. The $bla_{OXA-204}$ gene was located on a ca. 150-kb IncA/C-type plasmid, which also carried the bla_{CMY-4} gene. The $bla_{OXA-204}$ gene was associated with an IS*Ecp1* element, whereas the bla_{OXA-48} genes are usually associated with IS*1999*.

Among the different types of carbapenemases identified in *Enterobacteriaceae*, OXA-48 is one of the most commonly identified in many countries. OXA-48 is a class D carbapenem-hydrolyzing β-lactamase (CHDL) that was first identified in Turkey (1) and has subsequently been commonly reported, in particular, in the Middle East, North Africa, and Europe (2, 3). In addition, there have been recent reports of OXA-48-related enzymes, such as OXA-181, which has been identified in India (4) or in other countries with a link to India (5), as well as OXA-163 from Argentina (6). Those variants differ by few amino acid substitutions or deletions. OXA-48 and OXA-181 hydrolyze penicillins at a high level and carbapenems at a low level and spare broad-spectrum cephalosporins, whereas OXA-163 hydrolyzes broad-spectrum cephalosporins at a high level and carbapenems at a very low level. The bla_{OXA-48}-like genes are plasmid-borne and have been identified with insertion sequences involved in their acquisition and expression (7). The spread of the bla_{OXA-48} gene is mostly linked to the dissemination of a single IncL/M-type self-transferable plasmid (8).

Our study was initiated by the isolation of a *Klebsiella pneumoniae* strain showing reduced susceptibility to carbapenems and recovered from urine specimens of a single patient who had been hospitalized in Tunis, Tunisia. Identification of *K. pneumoniae* isolate 204 was performed by using the API 20E system (bioMérieux, La Balme-les-Grottes, France), and susceptibility testing was done by disc diffusion assays and interpreted according to the CLSI guidelines (9). *K. pneumoniae* 204 was resistant to all penicillins, to β-lactamase inhibitor–penicillin combinations, to broad-spectrum cephalosporins, and to ertapenem and was susceptible to imipenem and meropenem (Table 1). Double-disc synergy testing revealed that it expressed an extended-spectrum β-lactamase (ESBL). In addition, *K. pneumoniae* 204 was resistant to all aminoglycosides (netilmicin, amikacin, kanamycin, tobramycin, and gentamicin), fluoroquinolones, sulfonamides, and tetracycline (data not shown). The MICs of fosfomycin, colistin, and tigecycline, determined using E-tests, were at 32, 0.094, and 2 μg/ml, respectively. Whole-cell DNA of *K. pneumoniae* 204 was extracted using a QIAamp DNA Minikit and following the recommendations of the manufacturer (Qiagen, Courtaboeuf, France) and then used as the template under standard PCR conditions (10) with a series of primers designed for the detection of class A, B, and D β-lactamase genes (5, 11, 12). *K. pneumoniae* 204 expressed the ESBL CTX-M-14 that belongs to the CTX-M-9 cluster (13), together with ß-lactamases OXA-1, CMY-4, and SHV-1. In addition, *K. pneumoniae* 204 possessed a novel bla_{OXA-48}-like gene termed $bla_{OXA-204}$ (www.lahey.org/studies/).

Multilocus sequence typing, performed as described previously (14), identified *K. pneumoniae* 204 as a ST383 strain. This sequence type had been previously reported, corresponding to one isolate coproducing VIM-4, KPC-2, and CMY-4 from Greece (15) and to one isolate coproducing VIM-19 and a CTX-M-like β-lactamase from Sweden but with a Greek origin (16).

Shotgun cloning using HindIII-restricted genomic DNA and a HindIII-restricted pBK-CMV plasmid was performed as described previously (10). Recombinant plasmids were selected on Trypticase soy (TS) agar plates containing ticarcillin (100 μg/ml) and kanamycin (30 μg/ml). The resulting *Escherichia coli* (pBK-OXA-204) recombinant strain displayed a typical OXA-48-type phenotype, with high-level resistance to penicillins and penicillin-β-lactamase inhibitor combinations, reduced susceptibility to carbapenems, and susceptibility to expanded-spectrum cephalosporins (data not shown). Sequence analysis of the 7,688-bp cloned DNA fragment identified the $bla_{OXA-204}$ gene. Compared to OXA-48, OXA-204 exhibited two amino acid substitutions, namely, Gln98His and Thr99Arg (class D β-lactamase [DBL] nomenclature) (17). Sequencing of the insertion of recombinant plasmid pBK-OXA-204 revealed that the insertion sequence (IS) IS*Ecp1* was located immediately upstream of the $bla_{OXA-204}$ gene, as observed with the $bla_{OXA-181}$ gene (5) but in contrast to what is observed for bla_{OXA-48}, which is preceded by IS*1999* (2). Interestingly, the segment separating the $bla_{OXA-204}$ gene from the IS*Ecp1* element was 46 bp, whereas it was 128 bp for the $bla_{OXA-181}$ gene, suggesting that the two genes were not derived from each other by

Received 25 May 2012 Returned for modification 8 July 2012
Accepted 27 October 2012
Published ahead of print 31 October 2012
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doi:10.1128/AAC.01034-12

TABLE 1 MICs of β-lactams for *K. pneumoniae* 204, *E. coli* pTOPO-OXA-204, and *E. coli* pTOPO-OXA-48 in *E. coli* TOP10 or *E. coli* HB4 reference strains and *E. coli* TOP10(p204-B), *A. baumannii* CIP70.10(p204-B), *P. aeruginosa* PU21(p204-B), and reference strains *A. baumannii* CIP70.10 and *P. aeruginosa* PU21[c]

β-lactam[a]	MIC (μg/ml)										
	K. pneumoniae 204	*E. coli* TOP10 (pTOPO-OXA-204)	*E. coli* TOP10 (pTOPO-OXA-48)	*E. coli* TOP10 (p204-B)	*E. coli* TOP10	*E. coli* HB4 (pTOPO-OXA-204)	*E. coli* HB4 (pTOPO-OXA-48)	*E. coli* HB4	*A. baumannii* CIP70.10 (p204-B)	*A. baumannii* CIP70.10	*P. aeruginosa* PU21 (p204-B)	*P. aeruginosa* PU21
Amoxicillin	>256	>256	>256	>256	2	>256	>256	8	>256	32	>256	>256
Amoxicillin + CLA	>256	>256	>256	>256	2	>256	>256	8	>256	32	>256	>256
Ticarcillin	>256	>256	>256	>256	2	>256	>256	4	>256	8	>256	8
Ticarcillin + CLA	>256	>256	>256	>256	2	>256	>256	4	>256	8	>256	8
Piperacillin	>256	64	64	64	1	128	128	4	256	16	128	4
Piperacillin + TZB	128	64	64	64	1	128	128	4	256	8	128	4
Cephalothin	>256	8	8	>256	4	128	128	64	>256	32	>256	>256
Cefoxitin	128	2	2	32	2	256	256	256	>256	32	>256	>256
Ceftazidime	64	0.12	0.12	12	0.12	0.75	0.75	0.75	>256	4	24	1.5
Cefotaxime	128	0.25	0.25	12	0.06	1	1	0.38	>256	8	>256	>256
Aztreonam	32	0.06	0.06	4	0.06	0.38	0.38	0.38	64	32	8	1.5
Cefepime	16	0.25	0.25	0.25	0.06	1	1	0.5	16	2	2	1
Imipenem	0.5	0.5	0.5	0.5	0.06	32	32	0.25	8	0.25	8	1
Ertapenem	2	0.25	0.25	0.25	0.06	>32	>32	1	ND[b]	ND	ND	ND
Meropenem	0.5	0.06	0.06	0.06	0.01	32	32	0.25	4	0.25	32	0.75

[a] CLA, clavulanic acid at a fixed concentration of 4 μg/ml; TZB, tazobactam at a fixed concentration of 4 μg/ml.
[b] ND, data not determined because of the intrinsic resistance.
[c] Natural plasmid p204-B and recombinant plasmid pTOPO-OXA-204 expressed the OXA-204 β-lactamase.

FIG 1 Schematic map of the transposon Tn*2016* structure and the surrounding sequences. Open reading frames are shown as arrows or as horizontal boxes with an arrow indicating the orientation of the coding sequence. The IRL, IRR1, and IRR2 motifs are indicated (blackened base pairs are identical, whereas whitened base pairs are different), and target site duplications (A AATA or ACAAATAC) are represented by black bars. *orf1*, *orf2*, and *orf3* were similar to open reading frames encoding hypothetical proteins identified on IncA/C scaffolds.

mutations but had been mobilized independently through two distinct transposition processes from their natural progenitor, i.e., *Shewanella xiamenensis*. Noteworthy, detailed sequence analysis showed that IS*Ecp1* was truncated by another IS, namely, IS*Kpn15*. That latter element possessed a transposase belonging to the IS*66* family and showing 80% amino acid identity to that of IS*UnCu16*, an IS identified on plasmid pRSB113 isolated from a sewage treatment plant in Germany (18). IS*Kpn15* did not disrupt the −35 (TTGAAA) and −10 (TACAAT) promoter sequences located inside IS*Ecp1* and likely involved in the expression of the $bla_{OXA-204}$ gene, as demonstrated for $bla_{OXA-181}$ or bla_{CTX-M} genes (19). The $bla_{OXA-204}$ gene was part of a 4,420-bp potential transposon, named Tn*2016*, flanked by a 5-bp duplication of the target site (AAATA), being the signature of a transposition event, which occurred on the IncA/C scaffold (Fig. 1). This transposon was bracketed by two imperfect 14-bp inverted-repeat sequences, corresponding to the original inverted repeat left (IRL) of IS*Ecp1* and to a secondary inverted repeat right (IRR) IRR2 sequence showing identity of 6 of 14 bp with the IRL of IS*Ecp1*, in accordance with the IS*Ecp1*-mediated one-ended transposition process (Fig. 1). The identification of an 8-bp target site duplication (ACAAA TAC) at both extremities of IS*Kpn15* was likely the signature of a transposition event, which occurred in the IS*Ecp1*-$bla_{OXA-204}$-made tranposon (Fig. 1). Consequently, this result suggests that this truncation may have stabilized the Tn*2016* transposon on the IncA/C scaffold by disrupting the IS*Ecp1* transposase activity, therefore inactivating its one-ended transposition activity.

In order to compare the hydrolytic activity of OXA-204 to that of OXA-48, the corresponding genes were amplified using primers preOXA-48A and preOXA-48B, with DNAs of *K. pneumoniae* 204 and 11978 used as the templates, respectively (1, 5). The amplicons were cloned in the pCR-Blunt II-TOPO vector (Invitrogen, Cergy-Pontoise, France) and expressed in the *E. coli* TOP10 reference strain by following the manufacturer recommendations. Two *E. coli* recombinant strains, harboring recombinant plasmids pTOPO-OXA-48 and pTOPO-OXA-204, respectively, were obtained and verified by sequencing. They expressed OXA-48 and OXA-204, respectively, and displayed exactly the same β-lactam resistance pattern (Table 1). The specific activities of β-lactamases OXA-204 and OXA-48 for carbapenems, measured as described

TABLE 2 Specific activities of β-lactamases OXA-204 and OXA-48 for benzylpenicillin, broad-spectrum cephalosporins, and carbapenems[a]

Antimicrobial agent	Specific activity (mU/mg of protein ± SEM)	
	OXA-204	OXA-48
Benzylpenicillin	4,700 ± 330	4,700 ± 800
Imipenem	75 ± 7	80 ± 9
Ertapenem	2.5 ± 0.3	3 ± 0.4
Meropenem	2.8 ± 0.4	4 ± 0.7
Cefotaxime	100 ± 8	100 ± 8
Cefepime	7 ± 0.5	7 ± 0.5
Ceftazidime	NH[b]	NH

[a] In each case, three independent experiments were performed, and the mean and the standard error of the mean (SEM) were calculated.
[b] NH, hydrolysis not detectable.

previously (20) by using *E. coli* (pTOPO-OXA-204) and *E. coli* (pTOPO-OXA-48) as the templates, were very similar for benzylpenicillin, broad-spectrum cephalosporins, and carbapenems, suggesting that OXA-204 and OXA-48 possessed similar hydrolytic activities (Table 2). Then, these recombinant plasmids were electroporated into *E. coli* HB4, a porin-deficient strain (21), in order to evaluate the impact of their production in such an *E. coli* background. As expected, expression of both the $bla_{\text{OXA-204}}$ and $bla_{\text{OXA-48}}$ genes in *E. coli* HB4 conferred resistance to penicillins, β-lactamase inhibitor–penicillin combinations, and carbapenems at a quite high level (Table 1). The MICs of β-lactams for OXA-204 and OXA-48 were similar, confirming that OXA-204 and OXA-48 possessed very similar resistance patterns (Table 1).

Plasmid DNA analysis, performed as described previously (1), showed that *K. pneumoniae* 204 possessed three plasmids, namely, p204-A, p204-B, and p204-C, that were ca. 70, 150, and 180 kb in size, respectively (data not shown). Mating-out assays performed as described previously and using ertapenem (0.5 μg/ml) or ticarcillin (50 μg/ml) and sodium azide (100 μg/ml) for selection gave two types of transconjugants, *E. coli* J53(p204-A) and *E. coli* J53(p204-B). Transconjugant *E. coli* J53(p204-B) was resistant to penicillins, β-lactamase inhibitor–penicillin combinations, and broad-spectrum cephalosporins and showed decreased susceptibility to carbapenems (Table 1). It harbored the $bla_{\text{CMY-4}}$ and $bla_{\text{OXA-204}}$ genes. In addition, *E. coli* J53(p204-B) was resistant to tetracycline and to sulfamethoxazole-trimethoprim. Transconjugant *E. coli* J53(p204-A) displayed an ESBL phenotype and expressed the $bla_{\text{CTX-M-14}}$ gene. Using PCR-based replicon typing (PBRT) as described previously (22), plasmid p204-B was typed as an IncA/C and plasmid p204-A as an IncL/M.

This is the first identification of a $bla_{\text{OXA-48}}$-like gene on an IncA/C-type scaffold. Those plasmids possess a broad host range and are responsible for the spread of many resistance genes, in particular, the bla_{CMY}-like Ambler class C ß-lactamase genes (23). They have been detected worldwide, and carbapenemase genes such as $bla_{\text{NDM-1}}$ and $bla_{\text{VIM-4}}$ have recently been identified on that plasmid scaffold (24, 25). In our study, the broad host range of plasmid p204-B was confirmed by obtaining electrotransformants using *Acinetobacter baumannii* as the recipient strain and transconjugants using *Pseudomonas aeruginosa* as the recipient strain, as described previously (Table 1) (2, 5). PCR experiments confirmed the positivity for the $bla_{\text{OXA-204}}$ gene in the *A. baumannii* transformant and in the *P. aeruginosa* transconjugant. Plasmid DNA analysis further confirmed that the two recombinant strains harbored the same ca. 150-kb plasmid.

This study characterized the OXA-204 β-lactamase possessing a β-lactam resistance profile similar to that of OXA-48. The acquisition of the $bla_{\text{OXA-204}}$ gene was linked to IS*Ecp1*, as described for the $bla_{\text{OXA-181}}$ gene and many other acquired broad-spectrum β-lactamase genes, such as bla_{CMY} and $bla_{\text{CTX-M}}$ genes. Overall, the results of this study indicate that at least two different genetic backgrounds are associated with OXA-48-like carbapenemase-encoding genes in North Africa. Interestingly, the $bla_{\text{OXA-204}}$ gene was associated with the $bla_{\text{CMY-4}}$ gene on a widely diffused plasmid scaffold, giving rise to a successful genetic structure compromising the efficacy of all available β-lactams. Considering that IncA/C-type plasmids are known to spread efficiently, we might speculate that the diffusion of the $bla_{\text{OXA-204}}$ gene among Gram-negative bacteria will be significant.

The nucleotide sequence data reported in this work has been deposited in the GenBank nucleotide database under accession no JQ809466.

ACKNOWLEDGMENTS

This work was partially funded by a grant from the INSERM (U914) and the Université Paris XI, Paris, France, and by a grant from the European Community (TEMPOtest-QC, HEALTH-2009-241742).

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